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13. ABSTRACT *(Maximum 200 words)*

Paraneoplastic neurologic disease (PND) antigens are proteins normally expressed specifically in neurons that are expressed ectopically in tumors. It is believed that when expressed in tumors outside of the immunologically privileged nervous system, PND antigens stimulate the development of anti-tumor immunity, but also stimulate an autoimmune neuronal degeneration. We are studying PND's associated with breast cancer and immunity to the Nova and cdr2 antigens, assaying PND expression in clinical breast tumor specimens, and establishing animal models for the disorders. A prerequisite to studying the abnormal expression of PND antigens is an understanding of their normal expression. We have found that the Nova gene and antigen is normally restricted in expression to neurons. Strikingly, we have discovered that the cdr2 gene is widely expressed, but that its mRNA is under tight translational control, so that the cdr2 antigen is normally made only in neurons and (immune privileged) testes. This observation provides a novel insight to the biology of breast tumors, suggesting that they may dysregulate translational control to express abnormal proteins, and alters our approach to the anlysis of cdr2 expression in vivo. We have also made significant progress in generating gene constructs to generate mouse models of PND.

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INTRODUCTION:

Paraneoplastic neurologic disease (PND) antigens are proteins normally expressed specifically in the central nervous system (CNS) that are expressed ectopically in tumors; they have been termed onconeural antigens.[1] It is believed that their expression in tumors outside of the immunologically privileged CNS allows for their recognition by the immune system, and consequently a fortuitous anti-tumor immune response.[2] Tumor suppression is significant enough that the majority of PND patients present to clinicians asymptomatic from their tumors, but with newly developed neurologic symptoms. It is believed that the anti-tumor immune attack initiates the disorder, and that subsequently this immune response becomes competent to recognize neurons that normally express onconeural antigens, culminating in autoimmune neurologic disease. The most common tumors associated with PND are breast, ovarian and lung cancers.[2]

This model of the pathogenesis of PND is based largely on clinical observation. Studies of the expression and function of onconeural antigens in tumors and brain are lacking, as are animal models of their role in tumor biology and immunology. The purpose of the present work is to expand our understanding of the expression of onconeural antigens, and to develop an animal model for these syndromes. These studies are necessary to test and refine our model of the pathogenesis of these disorders. PND's provide examples of *bona fide* anti-breast tumor immunity in humans, and our hope is that the studies underway in this project will enable a detailed analysis of the mechanisms of successful anti-tumor immunity.

The methods of study in this project depend on the use of cloned genes [3-6] encoding the PND breast tumor antigens Nova and cdr2. The first specific aim is to assay clinical tumor specimens for the expression of PND genes and the presence of PND antigens. In some cases, it will also be appropriate to assay for co-factors that might be associated with the immune recognition of PND antigen (i.e. if we cannot establish a direct relationship between PND antigen expression and anti-tumor immunity). The second and third aims are complementary approaches aimed at establishing animal models for the breast tumor associated PND's, using breast tumor cell lines transfected with PND genes and transgenic mice making breast tumors expressing PND genes, respectively.

BODY:

Characterization of the Expression of PND Genes (Aim I):

One test of the model presented for the pathogenesis of the PND's is that the expression of onconeural antigens is normally restricted to neurons, allowing them to be recognized by the immune system as "foreign" antigens when ectopically expressed in breast tumors. A definitive analysis of the expression of the cdr2 gene has not previously been performed. We have now studied cdr2 mRNA expression by Northern blot analysis (Figure 1). Surprisingly, we find that cdr2 mRNA was abundant in all tissues, using either coding region or 3' UTR (gene specific) probes. Since this expression was particularly abundant in spleen, we confirmed expression of cdr2 mRNA in spleen by RT-PCR analysis (Figure 2), *in situ* hybridization of adult spleen with a cdr2 riboprobe (data not shown) and by sequencing a full length spleen cDNA, which was identical to the brain cdr2 cDNA (data not shown). We contrasted these results with an analysis cdr2 antigen expression by Western blot and immunohistochemical analyses, using antisera affinity purified against bacterially expressed cdr2 fusion protein (Figures 3 and 4; data not shown). These studies demonstrate that the cdr2 antigen is indeed expressed in a tissue-specific manner. The cdr2 protein and mRNA are found within discrete sets of neurons (primarily Purkinje cerebellar neurons, a few brainstem nuclei, and dorsal root ganglia; Figure 4 and data not shown), and is absent outside of the nervous system, with the exception of testes. Notably, normal breast tissue or ovarian tissue did not express cdr2 antigen. In addition, testes is notable as

the major immunologically isolated site outside of the nervous system. These studies suggest that the cdr2 gene is under strict translational control. The mRNA is widely expressed, but the protein expression is restricted to immunologically privileged sites. Our findings are reminiscent of observations of neuron-specific gene transcription mediated by transcriptional silencers outside of the nervous system.[7] Moreover, a direct precedent for our findings can be found in studies of the expression of the BTEB transcription factor. BTEB mRNA is made in many tissues but, due to 5'-UTR translational control elements, the protein is made only in brain and testes.[8]

The observation that cdr2 antigen expression is tissue specific is consistent with the current model of PND pathogenesis, and is particularly significant for the current project. It indicates that the study of cdr2 mRNA in breast and ovarian tumor tissues, as originally proposed, is not the appropriate level of analysis. Rather, an analysis of cdr2 antigen expression, as assayed by Western blot and immunohistochemistry, will be the relevant focus of study for this antigen. Consistent with these observations, our preliminary analysis of cdr2 mRNA expression by RT-PCR analysis of non-PND breast tumor tissues revealed the presence of cdr2 mRNA; however, interpretation of this as ectopic expression in non-PND tissues now awaits repeating this analysis with Western blot analysis. To facilitate these studies, we will use PND antisera that has been affinity purified using a cdr2 clone, and, in addition, we have generated a polyclonal rabbit antisera specific for the cdr2 antigen (data not shown).

Preliminary work indicated that the expression of the Nova antigen was normally restricted to neurons using PND antisera to assay protein expression.[6] However, since we have found evidence for more than one Nova gene family member (Darnell et al., unpublished data), we have pursued this observation at the level of expression of the Nova-1 gene, using a gene specific probe for *in situ* hybridization. These studies (Figure 1 and data not shown) confirm that the Nova-1 gene and antigen are restricted to neurons at all points during the normal development of mice, and support the model for the pathogenesis of the PNDs outlined in the introduction. Studies of Nova mRNA expression in non-PND breast and ovarian tumor tissue is now appropriate; mRNA has been prepared from 50 samples in a laboratory physically separated from our own (to avoid contamination that would affect RT-PCR analysis), and RT-PCR analysis of Nova mRNA expression is currently underway.

Animal Models of Breast Tumor Immunity #1: PND transfection of tumor cell lines (Aim 2):

These experiments will attempt to establish anti-breast tumor immunity in mice to a breast tumor cell line (RAC) transfected with the PND breast tumor antigens Nova and cdr2. As a prelude to setting up these cell lines, we assayed the parental RAC cells for Nova and cdr2 expression (see below). To establish PND gene expression constructs, full length Nova and cdr2 cDNA's have been amplified by PCR, sequenced in their entirety to ensure the integrity of the PCR reaction. A total of 3 complete cdr2 and 3 complete Nova PCR (Pfu polymerase was used to increase fidelity of the reaction) amplified cDNA's were sequenced on both strands, yielding in each case one clone matching the native sequence. These were subcloned into mammalian expression vectors containing a selectable marker (the E. Coli gpt gene; Pharmacia) and transfected into RAC cells.

Figure 5 shows a Western blot analysis of Nova-1 protein expression in parental vs. Nova-transfected RAC cells. Non-transfected parental cells express no detectable Nova-1 antigen, while transfected cells express readily detectable protein levels. Similar results are found with the cdr2 antigen (data not shown). These experiments demonstrate the fidelity of the expression vector clones, and the suitability of comparing the tumorigenicity of parental vs. transfected RAC cells in Balb/C mice as proposed.

Animal Models of Breast Tumor Immunity #2: PND expression in transgenic breast tumors (Aim 3):

In the proposed experiments, we plan to utilize currently available breast tumor transgenic mice (MMTV-wnt, neu and myc) to test the role of PND antigens in anti-tumor immunity. To adequately control these studies, we plan to adoptively transfer transgenic breast tumors that either do or do not express PND antigens into naive syngeneic mice.

The background work to establish such experiments includes an analysis of transgenic breast tumors for endogenous PND antigen expression, followed by construction of MMTV-driven PND expression vectors. To date, we have obtained MMTV-myc and MMTV-wnt breast tumor tissue (from Dr's. P. Leder and H. Varmus, respectively) for analysis of PND expression. We have analyzed these tumors for Nova antigen expression, and find no evidence that they express this antigen (Figure 6). Initial studies indicated that each tumor type expressed cdr2 mRNA (data not shown); however, given our results (above) indicating that cdr2 is under tight translational control, we are currently evaluating cdr2 antigen expression in these tumors by Western blot analysis.

Full length Nova and cdr2 cDNA constructs amplified by PCR and sequenced in shuttle vectors (above) were cloned into the MMTV-promoter mammalian expression vector pMSG (Pharmacia). The integrity of these constructs has been confirmed by transfection into RAC cells and analysis of protein expression by Western blot.

CONCLUSIONS:

The completed research demonstrates that the Nova and cdr2 PND antigens are both normally expressed in immune-privileged sites. This supports our general model for the pathogenesis of the breast tumor immunity seen in PND patients. That is, the immune system appears to recognize these antigens in the context of breast tumor cells as foreign antigens; their normal restriction to neurons (and testes for cdr2) sequesters them from immune surveillance and prevents their recognition as self antigens. Strikingly, we have found that the mechanisms for sequestering Nova and cdr2 antigens to neurons are very different. Nova mRNA and protein are found to be sequestered to neurons at all developmental times. In contrast, cdr2 mRNA is widely expressed in various tissues; within the nervous system its expression is fairly restricted to Purkinje cerebellar neurons, brainstem neurons and dorsal root ganglia, but outside the nervous system many tissues express the gene. Tight restriction of cdr2 antigen expression to neurons and testes is demonstrated by Western blot and immunohistochemical analysis, leading to the conclusion that translational regulatory mechanisms restrict the expression of cdr2 to immune-privileged sites.

These observations have important implications for breast tumor biology, suggesting for example that tumors that ectopically express cdr2 antigen have dysregulated translational control mechanisms. Such dysregulation has not been previously observed in breast tumors, and suggests a possible target for therapeutic intervention. Moreover, these observations recommend changes in our future work to better address the issue of the induction of ectopic PND antigen expression in tumors. Specifically, we are interested in analyzing normal and PND breast tumors for the expression of Nova and cdr2. We originally envisioned focusing on the analysis of mRNA expression using RT-PCR in such experiments. Given our current understanding of cdr2 regulation, however, we will now switch our plan to include a careful analysis of both mRNA and protein expression (analyzed by Western blot analysis) in these tumors.

Finally, we have made significant progress in generating and testing gene constructs for the construction of animal models of PND, and have performed important controls assaying Nova and cdr2 protein expression in tumors obtained from transgenic breast tumor mice and breast tumor cell lines. One recommended change in our constructs derived from our

work on cdr2 is that all expression constructs will include coding sequence without any 5'-UTR sequence. In order to obtain efficient ectopic expression of cdr2 protein in our animal models, we need to circumvent potential down regulatory translational control elements in the cdr2 mRNA, and, based on the literature [8], we predict that they reside in the 5'-UTR.

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Figure 1

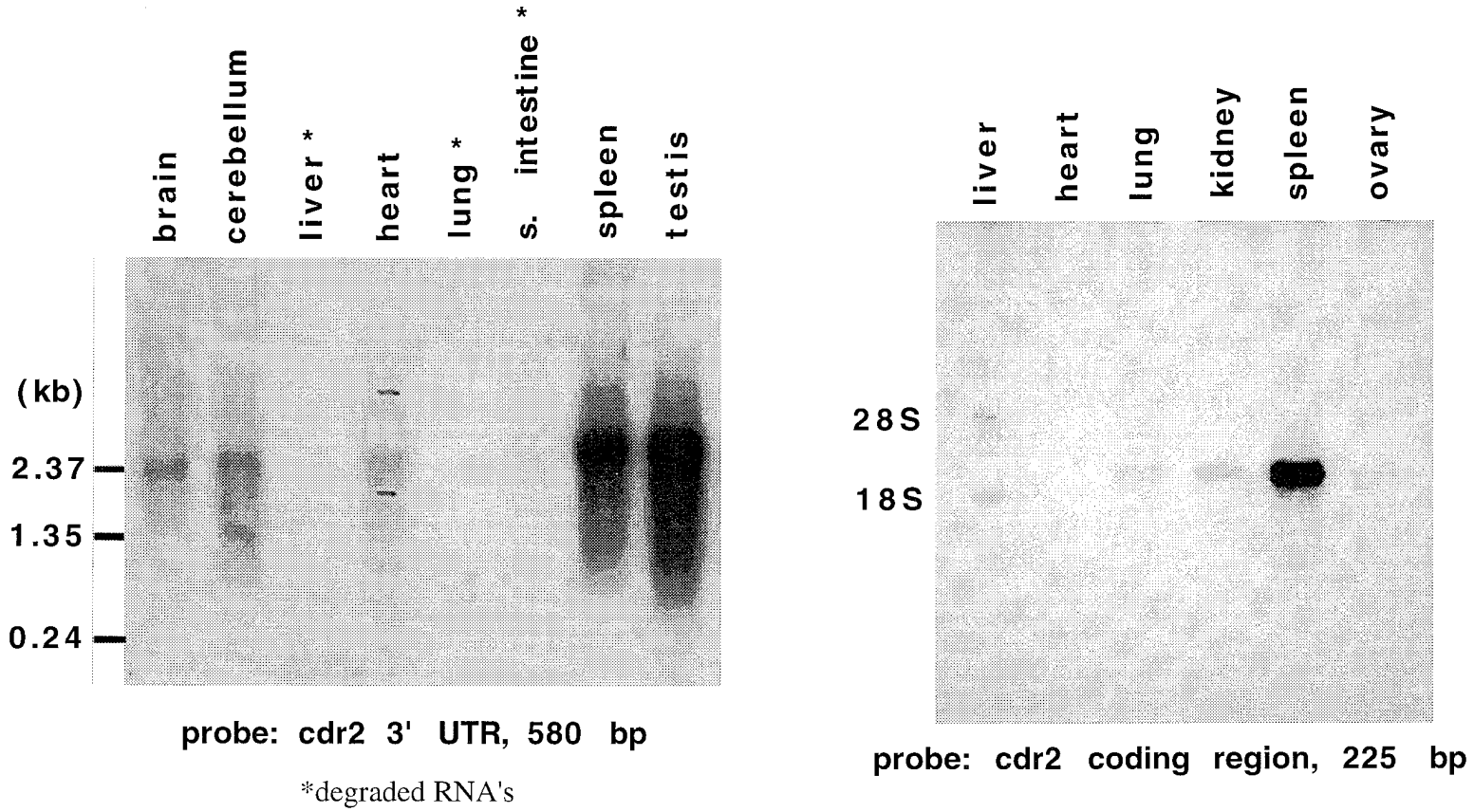

Figure 1. Analysis of cdr2 mRNA. Total RNA was extracted from the indicated mouse tissues, run on denaturing formaldehyde gels, transferred to nitrocellulose and probed with the indicated gene-specific probes. cdr2 mRNA of approximately 2.4kb could be detected in all tissues, with particularly robust expression in spleen, testes, brain, kidney, heart and ovary. RNA from starred samples (liver, lung, small intestine) was visibly degraded by EtBr staining; independent blots confirmed cdr2 expression in lung and small intestine.

Figure 2

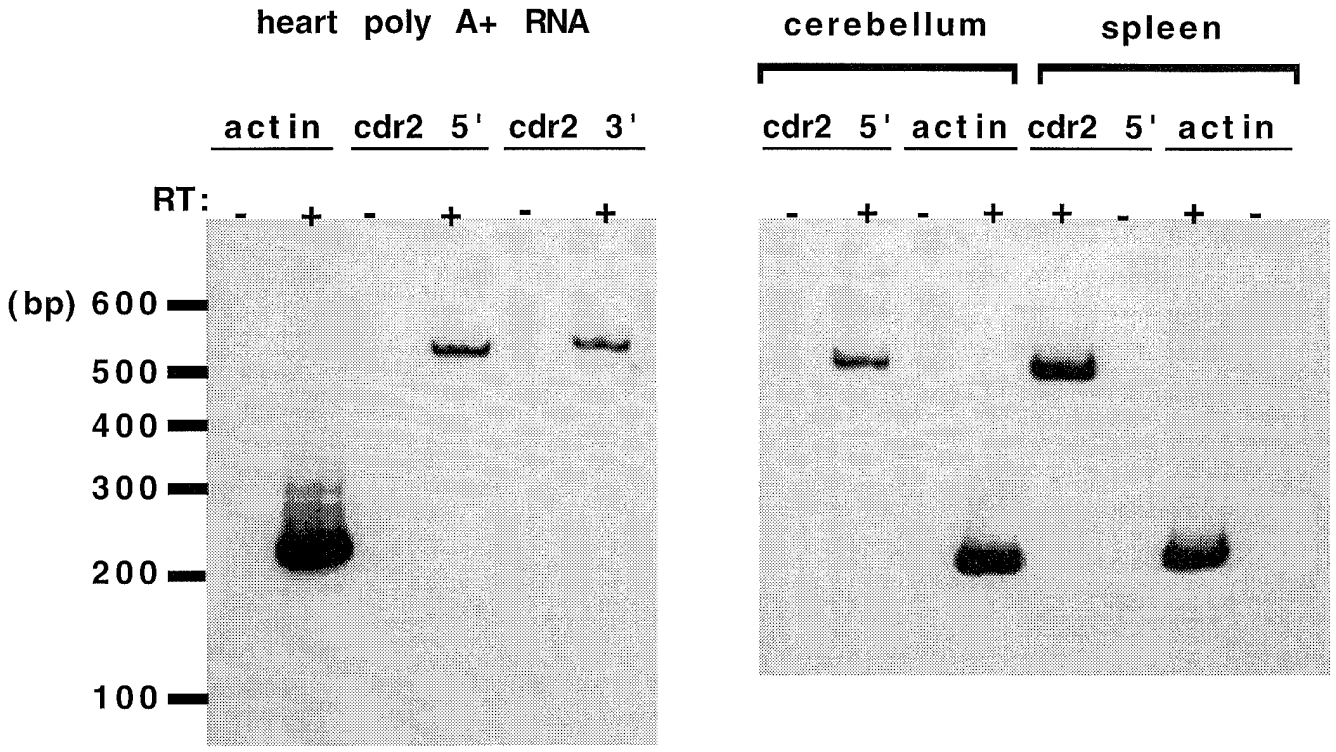

Figure 2. RT-PCR analysis of cdr2 mRNA in neuronal and non-neuronal tissues. Total or poly A+ RNA was prepared from the indicated tissues. Duplicate samples underwent reverse transcription and PCR with the cdr2 primers or actin control primers, in the absence or presence of reverse transcriptase (to control for contaminating DNA). cdr2 mRNA yielded RT-dependent PCR products of the expected size with cdr2 primers from heart, cerebellum and spleen mRNA.

Figure 3

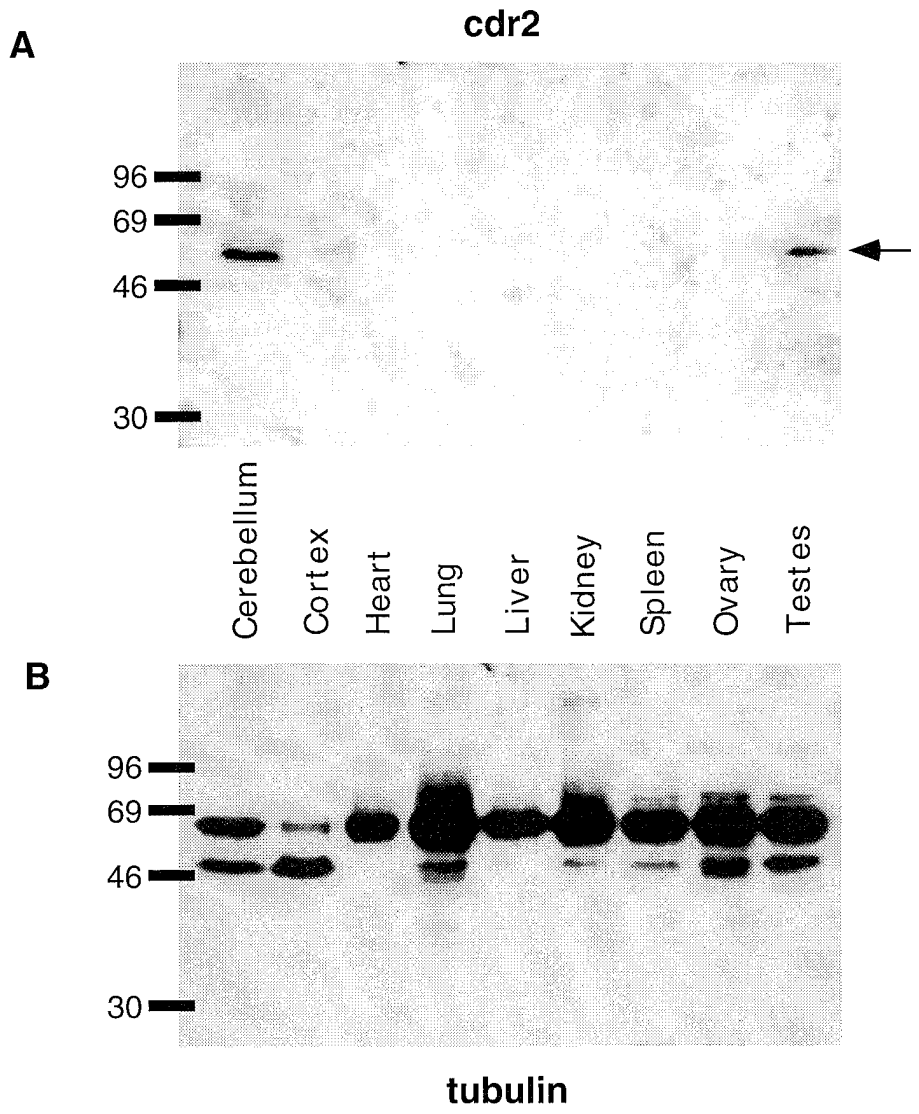

Figure 3. Western blot analysis of cdr2 antigen expression in mouse tissues. A. Extracts of normal mouse tissues were run on SDS-PAGE and probed with cdr2 (Yo) paraneoplastic disease antiserum. cdr2 antigen of the expected size (~52kD; arrow) was detected in cerebellum and spleen, with a fainter signal evident in cortex; there is no detectable antigen in non-neuronal tissues with the exception of testes. B. The same blot was stripped and reprobed with an antibody to tubulin, demonstrating the presence of approximately equal amounts of protein in each lane.

Figure 4

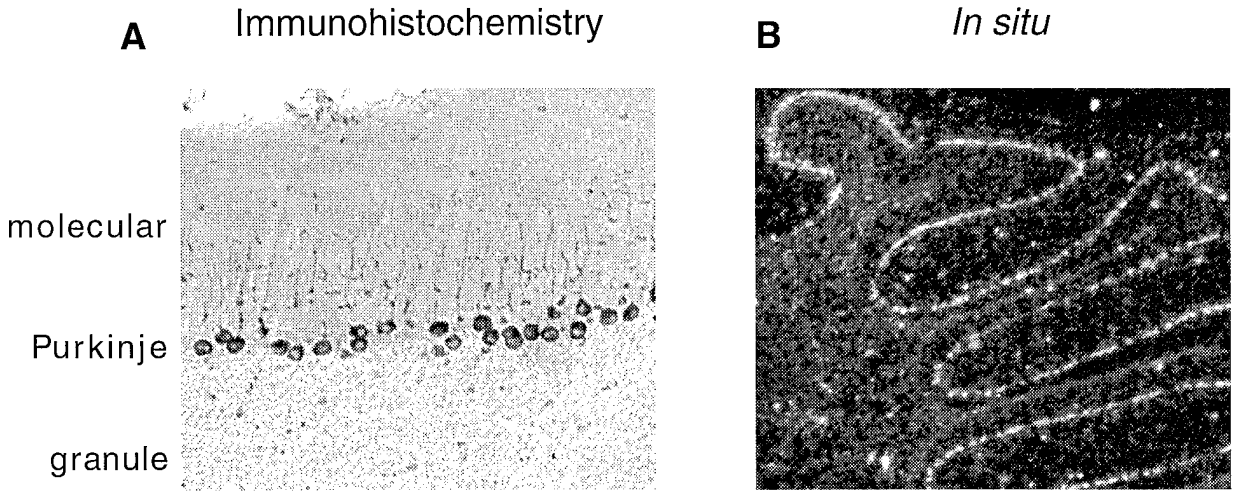

Figure 4. Neuron-specific expression of cdr2 protein and mRNA. A. An 8μM frozen section of mouse cerebellum was probed with cdr2 (Yo) PND antiserum and visualized by avidin-biotin peroxidase immunohistochemistry. Expression of the cdr2 antigen is restricted to Purkinje neurons in the cerebellum. B. An 8μM frozen section of mouse cerebellum was probed with a ^{33}P-labeled cdr2 antisense riboprobe and visualized by dark field microscopy. The cdr2 mRNA is restricted to Purkinje neurons in the cerebellum.

Figure 5

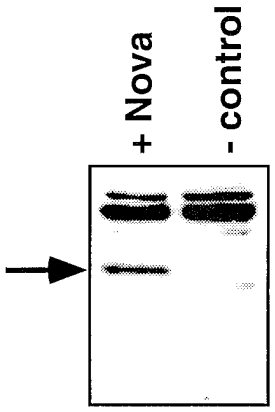

Figure 5. Western blot analysis of RAC breast tumor cell lines stably transfected (Eco gpt selection) with a Nova-1 expression vector (+ Nova) or a parental plasmid vector (-control). Extracts of RAC cells were run on Western blots and probed with Nova paraneoplastic disease antiserum. Nova antigen is readily detected in Nova-1 transfected cells but not in mock transfected cells.

Figure 6

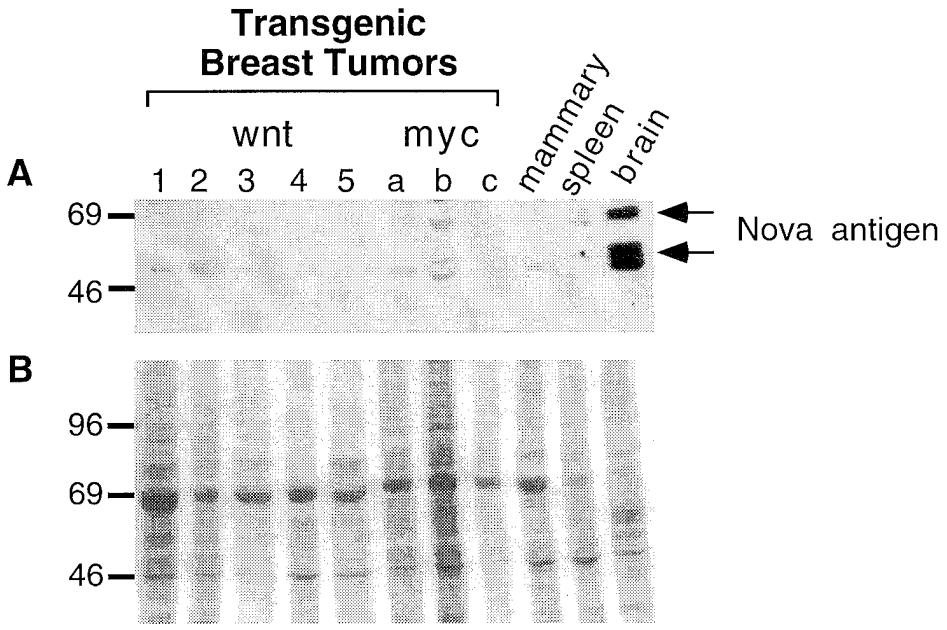

Figure 6. Western blot analysis of Nova antigen expression in transgenic breast tumor tissues. A. Extracts of breast tumors dissected from MMTV-wnt or MMTV-myc transgenic mice were run on Western blots and probed with Nova paraneoplastic disease antiserum. Nova antigen is readily detected in brain extracts, but is not evident in normal mouse mammary or spleen, nor in transgenic breast tumor tissues. B. Coomassie blue stain of samples probed in (A), demonstrating that an approximately equal amount of protein was loaded in each lane.